(12) UK Patent Application (19) GB (11) 2 345 851 (13) A

(43) Date of A Publication 26.07.2000

(21) Application No 0000984.5

(22) Date of Filing 18.01.2000

(30) Priority Data
(31) 99008443 (32) 18.01.1999 (33) GB

(71) Applicant(s)
Gavin Paul Vinson
37 Birchwood Ave, MUSWELL HILL, London, N10 3BE, United Kingdom

John Puddefoot
10 Sunnyside Gardens, UPMINSTER, Essex, RM14 3DS, United Kingdom

George Margetts
44 Broomfield Drive, BILLINGSHURST, West Sussex, RH14 9TN, United Kingdom

(72) Inventor(s)
Gavin Paul Vinson
John Puddefoot
George Margetts

(51) INT CL[7]
A61K 31/58 , A61P 35/00

(52) UK CL (Edition R)
A5B BHA B180 B24Y B240 B242 B243
U1S S1313

(56) Documents Cited
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Anticancer Res., 15(4), 1349-54, (1995) J. Steroid Biochem. Mol. Biol., 37(2), 231-6, (1990) Cancer Treatment Reports, 71(12), 1197-1201, (1987) Cancer Chemo. Pharmacol., 10(3), 158-60, (1983)

(58) Field of Search
UK CL (Edition R) A5B
INT CL[7] A61K 31/58
ONLINE: CAS-ONLINE, EPODOC, JAPIO, TXTE, WPI

(74) Agent and/or Address for Service
Christopher Barry Wood
The Cedars, Hollybush Hill, STOKEPOGES, Bucks, SL2 4QN, United Kingdom

(54) Abstract Title
Modulating hormone receptor sites in cells using trilostane

(57) Certain steroid compounds which are 2α-cyano-4α, 5α-epoxy-3-oxo steroids notably trilostane, act on specific isoforms of the oestrogen receptor in human and animal cells. The compounds are used to treat certain conditions in hormone-sensitive organs such as but not exclusively breast, prostate, central nervous system, heart, blood vessels and uterus. The invention shows that trilostane and related compounds selectively modulate ligand binding to the oestrogen receptor and in particular to oestrogen receptor beta.

Also claimed are particulate forms of these compounds which consist of particles having a mean equivalent sphere volume diameter of 12 μm or less and 90% having a particle size less than 50 μm.

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Fig. 1

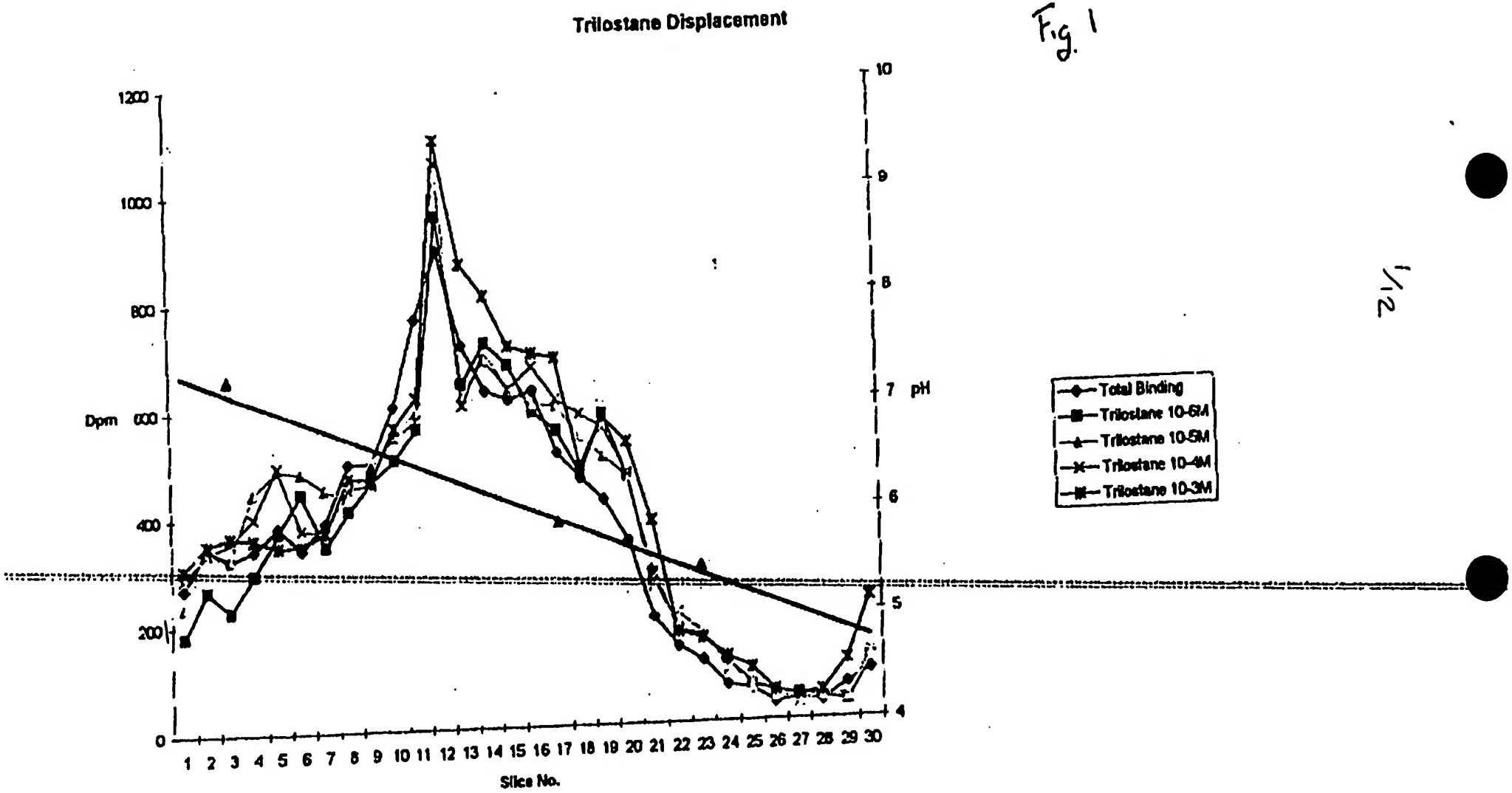
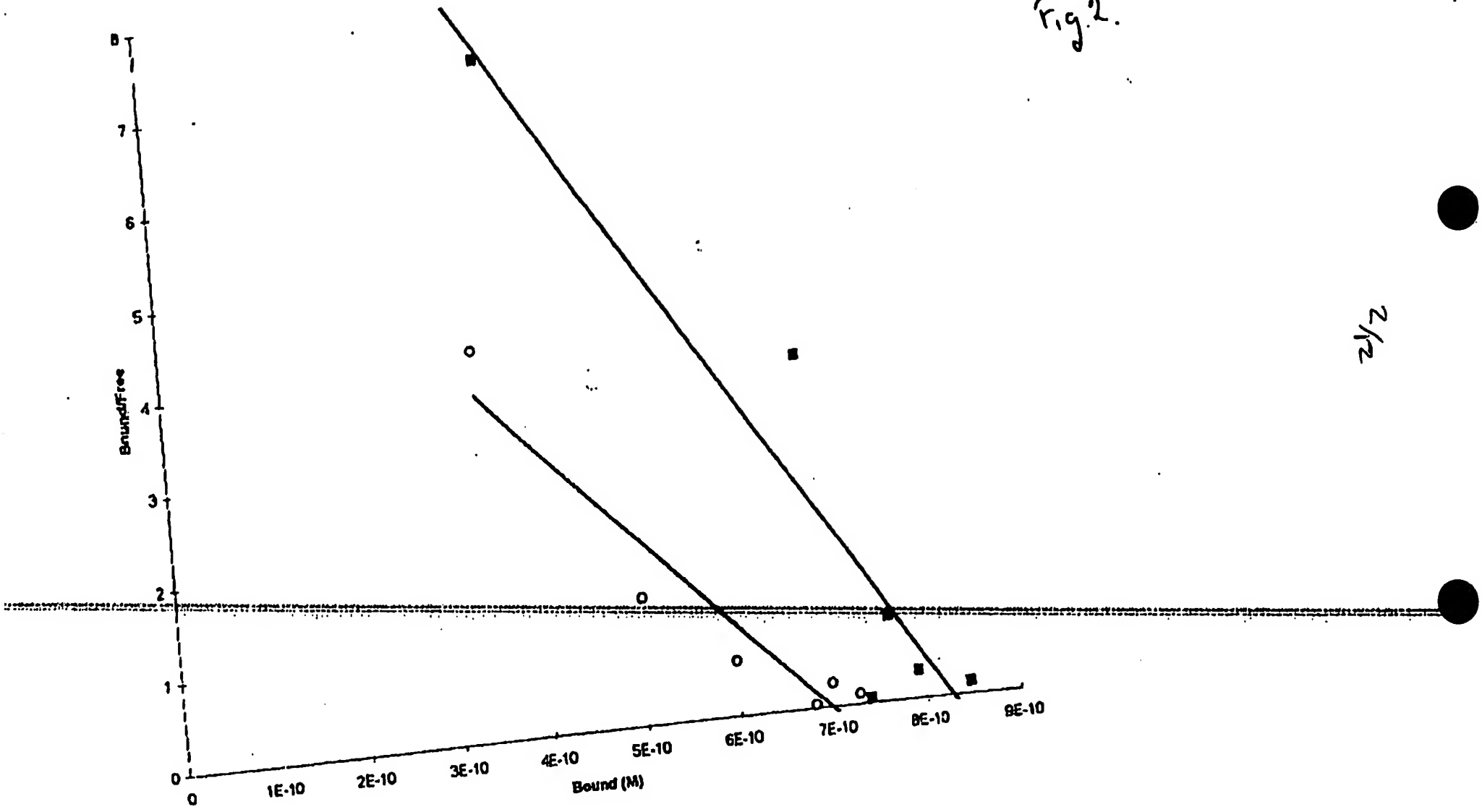

Trilostane Displacement
Dpm
pH
Slice No.
Total Binding
Trilostane 10-6M
Trilostane 10-5M
Trilostane 10-4M
Trilostane 10-3M

Fig. 2.


B
7
6
5
4
3
2
1
0
Bound/Free
0
1E-10
2E-10
3E-10
4E-10
5E-10
6E-10
7E-10
8E-10
9E-10
Bound (M)

2/2

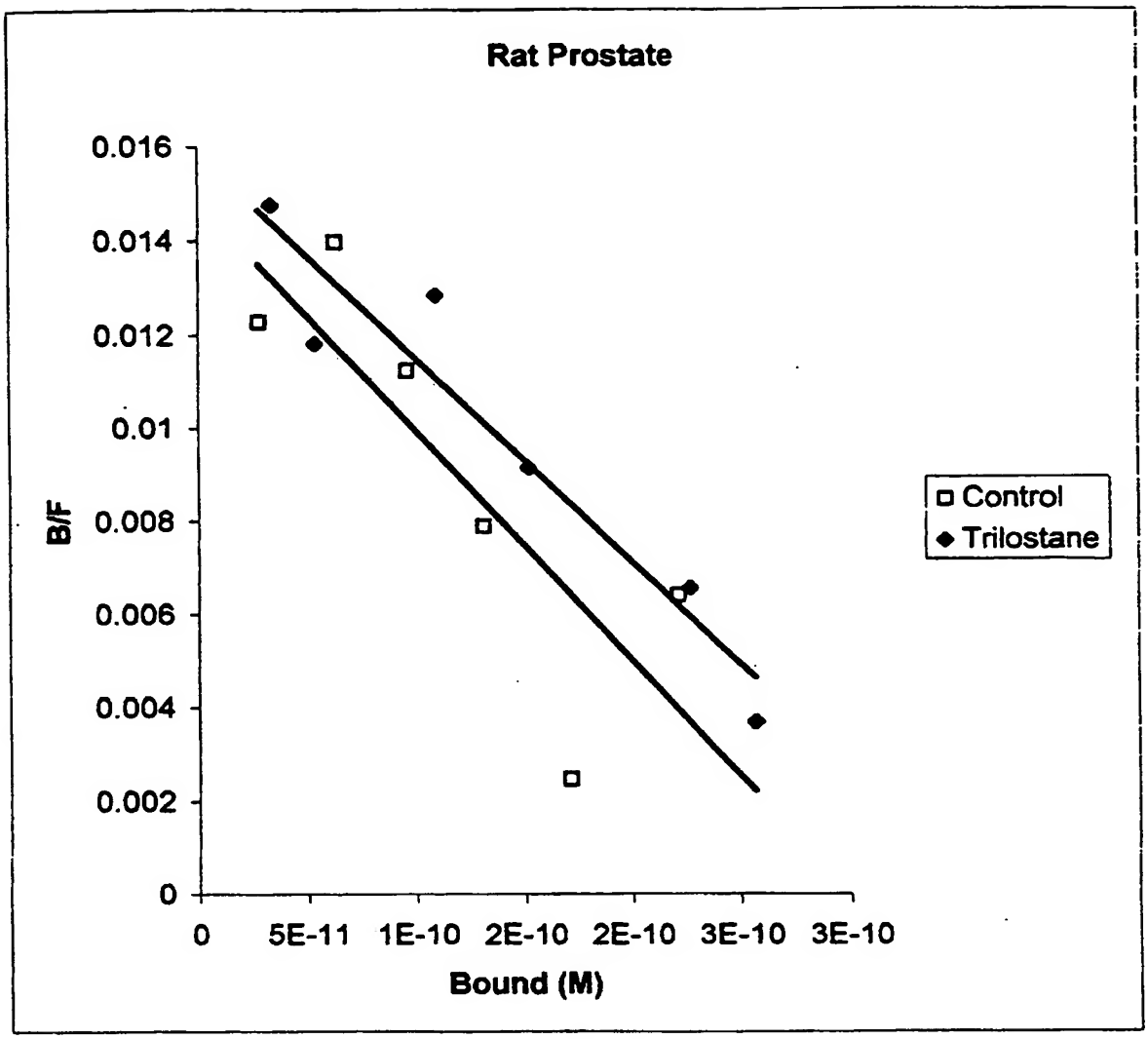

Rat Prostate
0.016
0.014
0.012
0.01
0.008
0.006
0.004
0.002
0
B/F
0
5E-11
1E-10
2E-10
2E-10
3E-10
3E-10
Bound (M)
Control
Trilostane

Figure 3 (a)

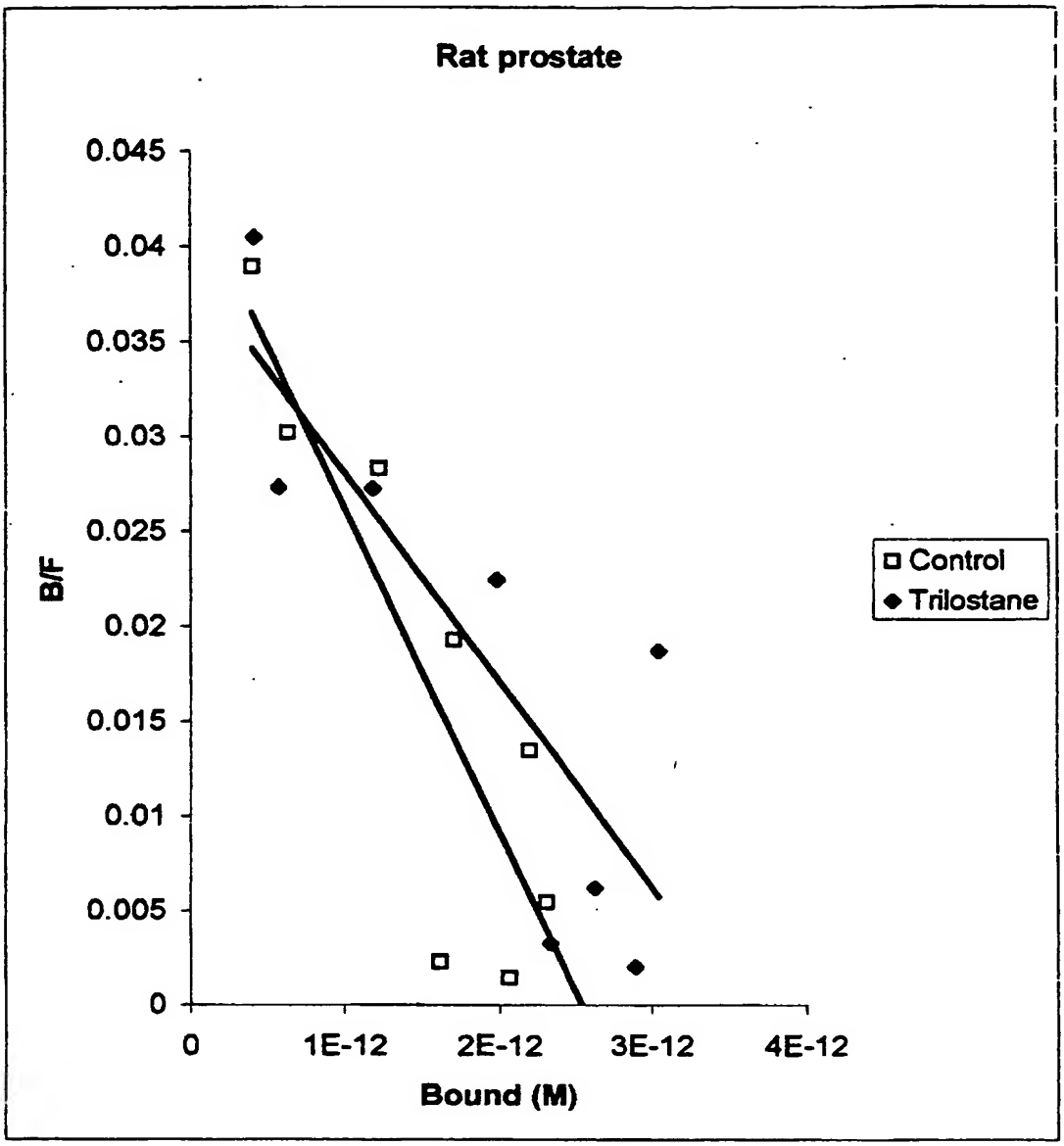

Rat prostate
0.045
0.04
0.035
0.03
0.025
0.02
0.015
0.01
0.005
0
B/F
0
1E-12
2E-12
3E-12
4E-12
Bound (M)
Control
Trilostane

Figure 3 (b)

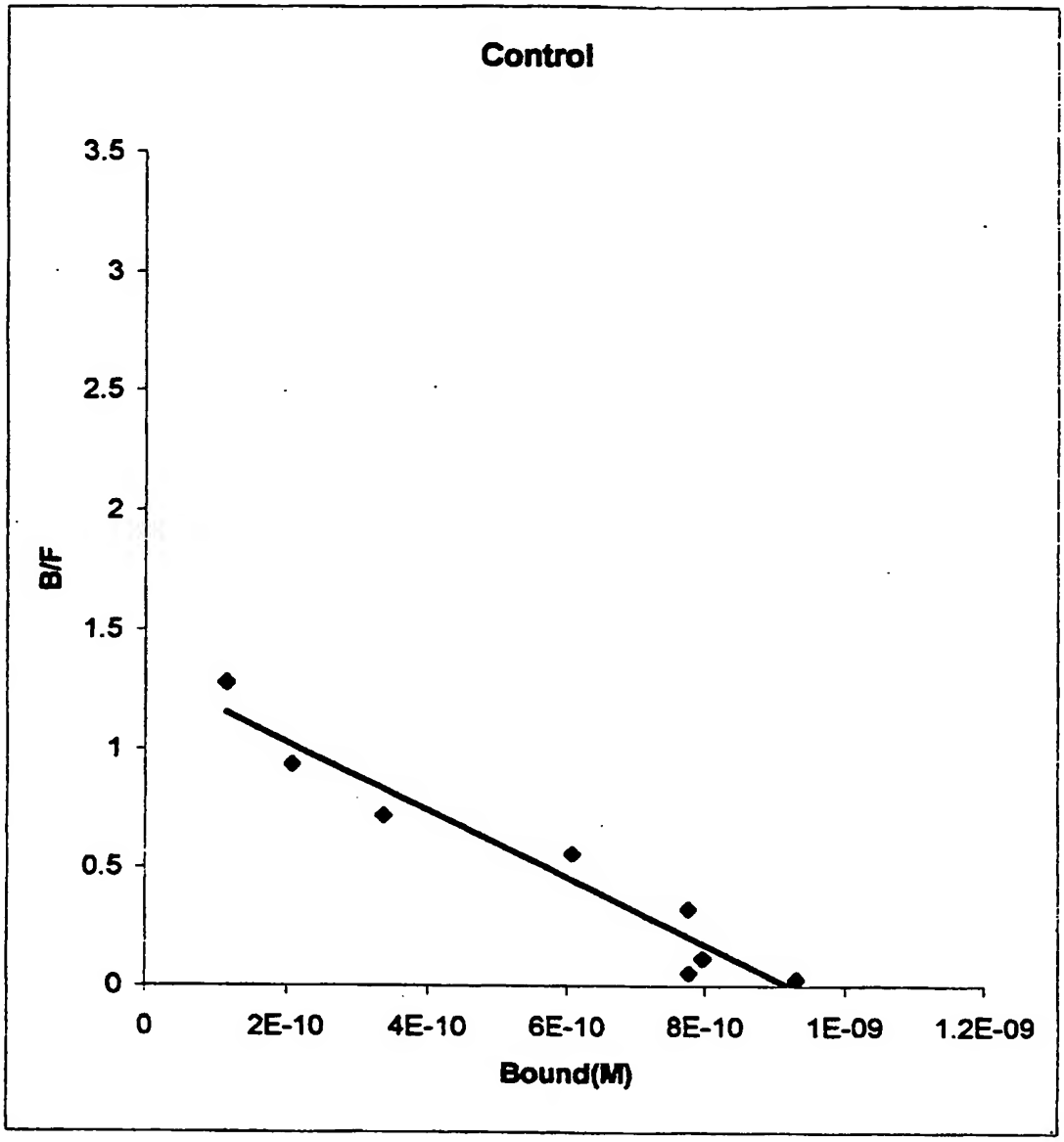

Control
3.5
3
2.5
2
1.5
1
0.5
0
B/F
0
2E-10
4E-10
6E-10
8E-10
1E-09
1.2E-09
Bound(M)

Figure 4 (a)

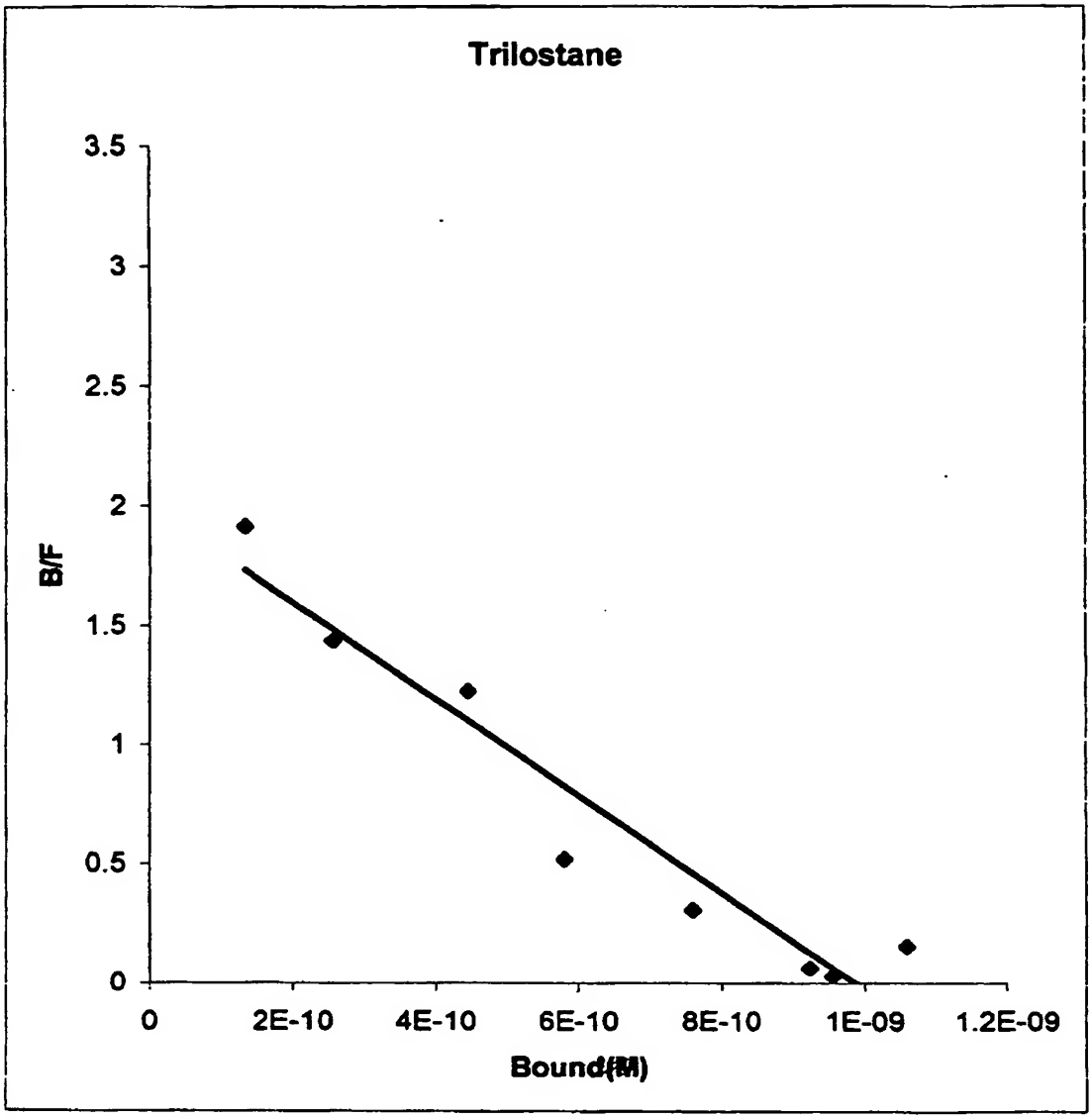

Trilostane
3.5
3
2.5
2
1.5
1
0.5
0
B/F
0
2E-10
4E-10
6E-10
8E-10
1E-09
1.2E-09
Bound(M)

Figure 4 (b)

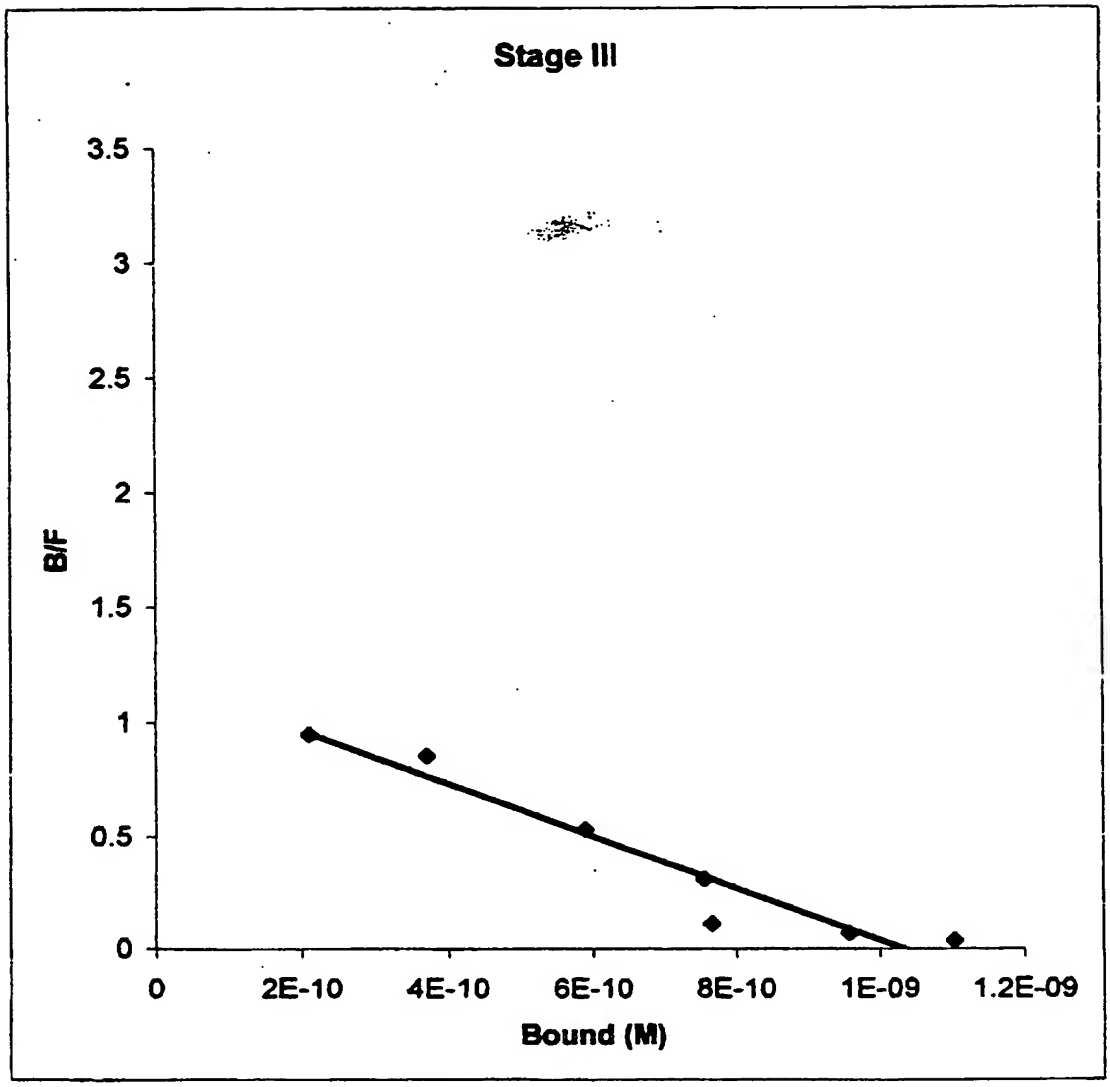

Stage III
B/F
3.5
3
2.5
2
1.5
1
0.5
0
0
2E-10
4E-10
6E-10
8E-10
1E-09
1.2E-09
Bound (M)

Figure 4 (c)

8/12

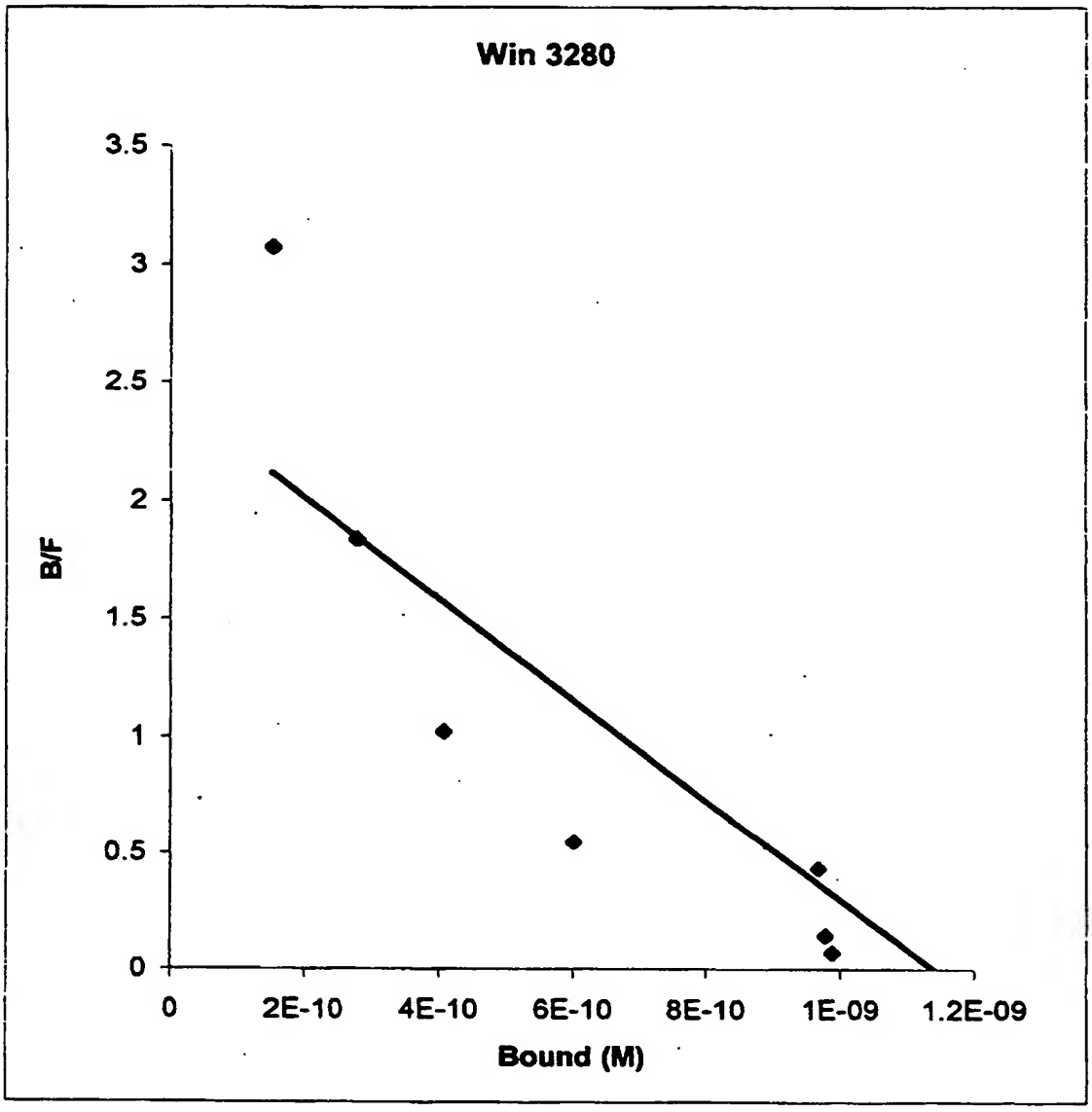


Figure 4 (d)

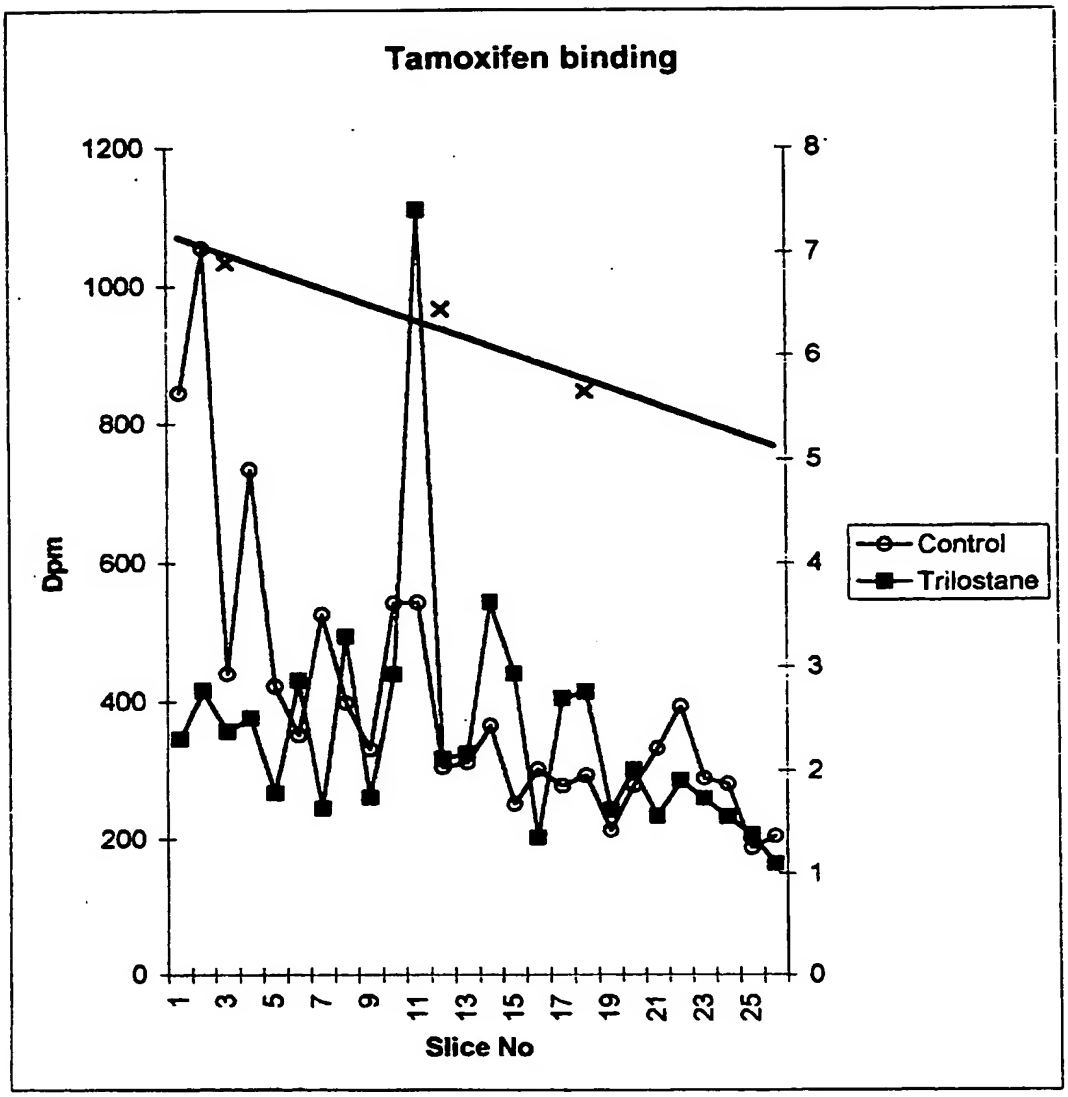

Tamoxifen binding
1200
1000
800
600
400
200
0
Dpm
8
7
6
5
4
3
2
1
0
1
3
5
7
9
11
13
15
17
19
21
23
25
Slice No
Control
Trilostane

Figure 5 (a)

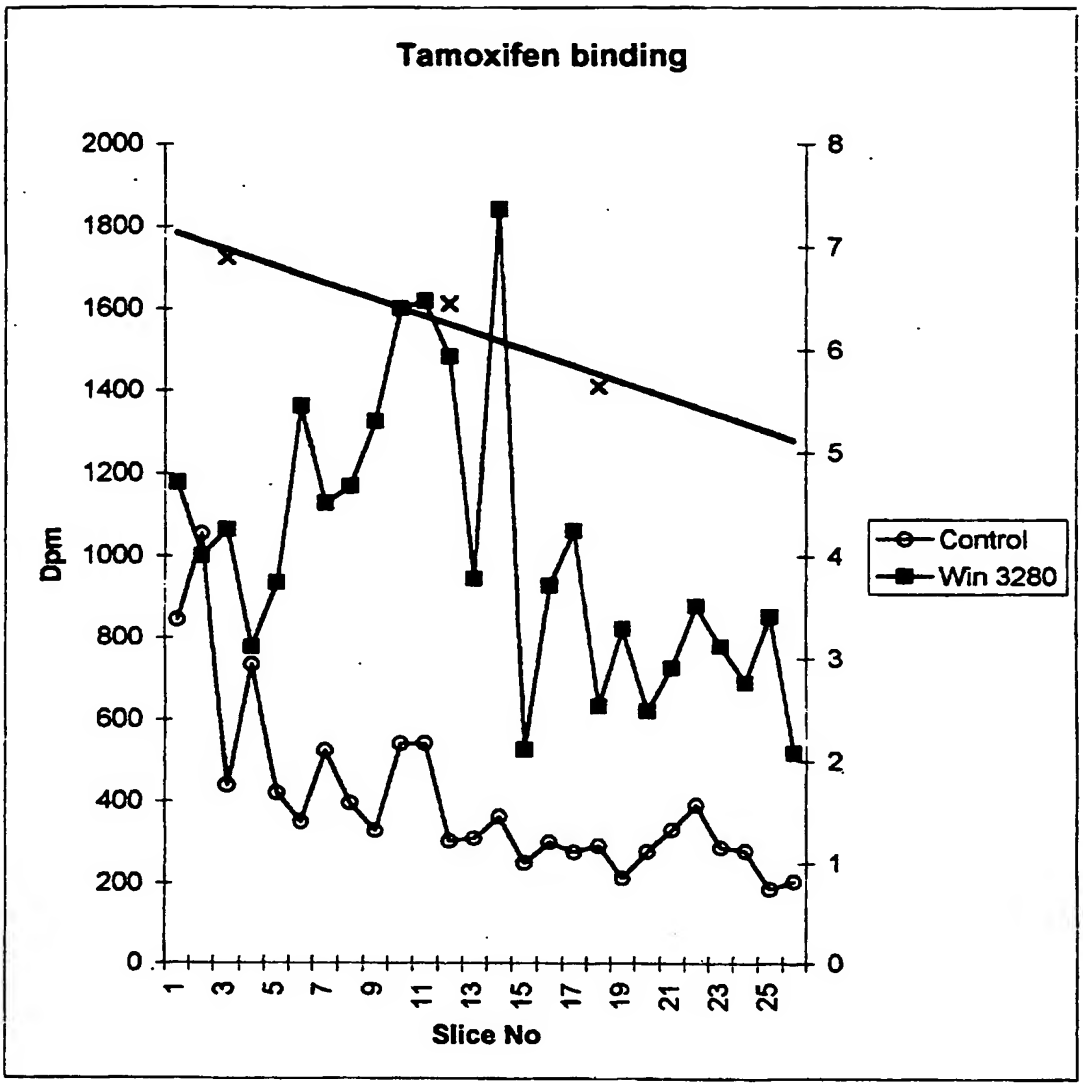

Tamoxifen binding
Dpm
2000
1800
1600
1400
1200
1000
800
600
400
200
0
8
7
6
5
4
3
2
1
0
1
3
5
7
9
11
13
15
17
19
21
23
25
Slice No
Control
Win 3280

Figure 5 (b)

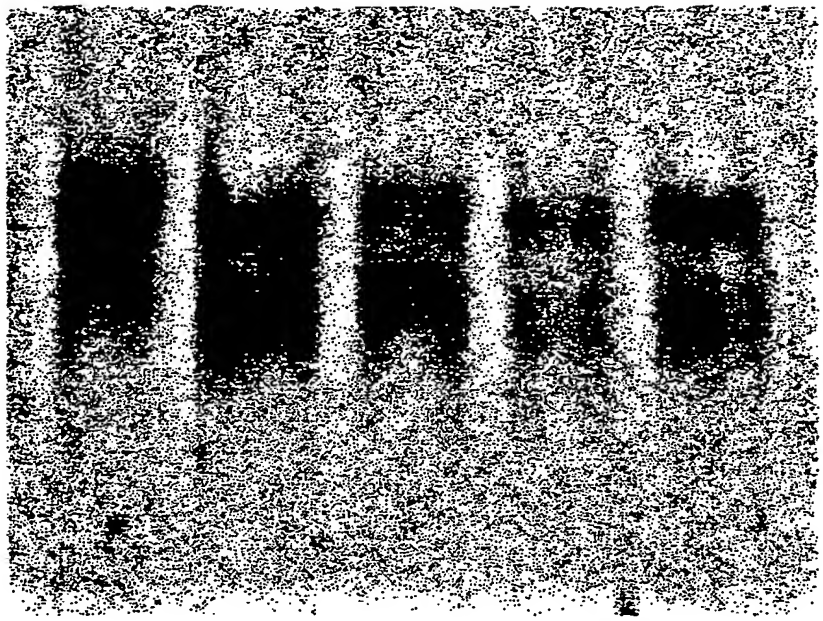


Figure 6

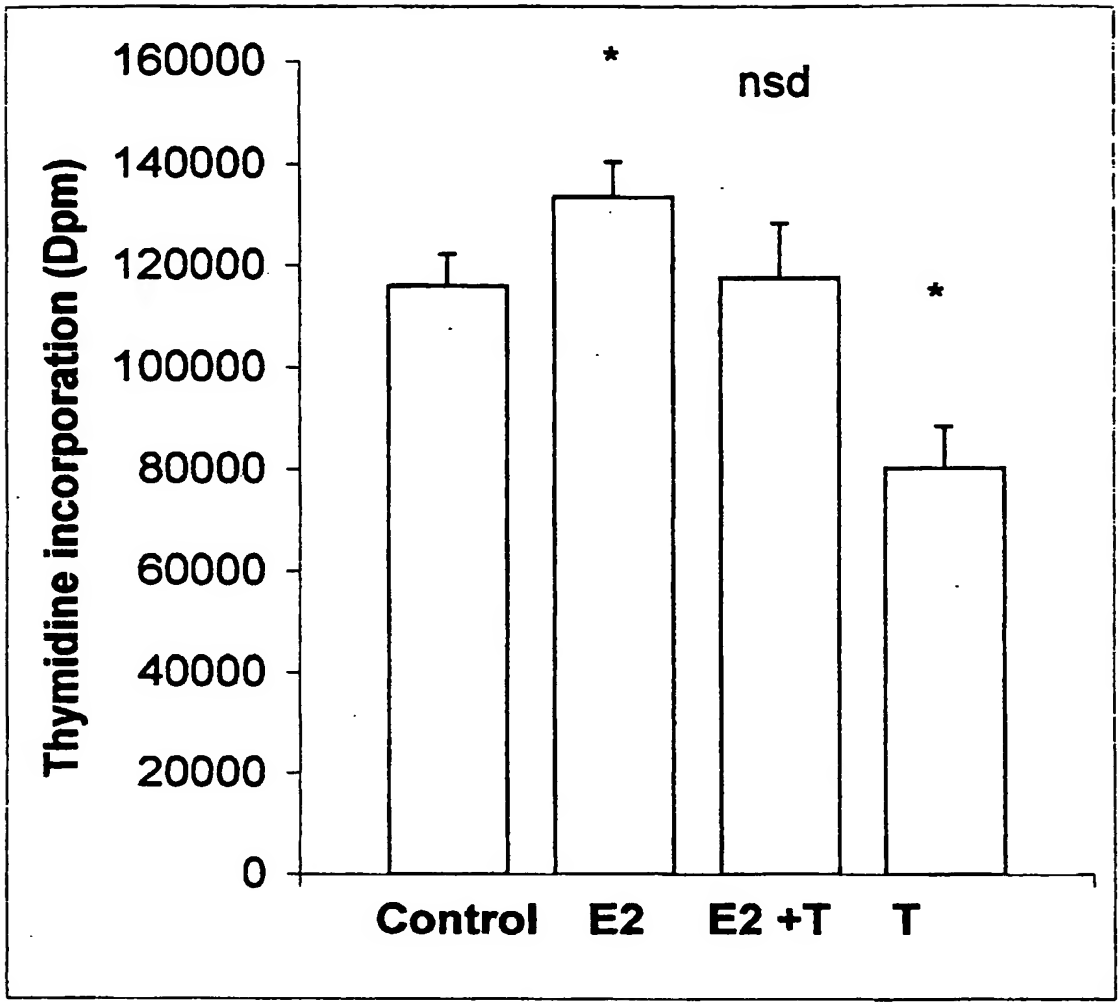

160000
140000
120000
100000
80000
60000
40000
20000
0
Thymidine incorporation (Dpm)
*
nsd
*
Control
E2
E2 +T
T

Figure 7

A NOVEL METHOD OF MODULATING HORMONE RECEPTOR AND OTHER RECEPTOR SITES IN HUMAN AND ANIMAL CELLS USING TRILOSTANE AND RELATED COMPOUNDS AND THE USE OF THESE COMPOUNDS TO TREAT CERTAIN DISEASES.

SUMMARY

The present invention relates to a method whereby certain steroid compounds, and in particular to pharmaceutical compositions comprising an active ingredient selected from those steroid compounds, modulate the oestrogen receptor(s) on human and animal cells. The modulation by these compounds is directed at specific isoforms of the oestrogen receptor(s). The effect of such modulation is to change the binding characteristics of ligands, and in particular oestrogen, to the receptor site.

The selective modulation of steroid receptors in human and animal cells is the current basis for the hormonal treatment of various cancers including but not exclusively breast and prostate cancer in man. The present invention describes a novel mode of action for certain steroid compounds and the potential use of these compounds alone or in combination with other agents to treat hormone-dependant cancers.

BACKGROUND

Oestrogen is a vital hormone in the human and animal body and plays a crucial role in the development and maintenance of organ function. Its most well known actions are in promoting lactation in the breast, regulating the production of cholesterol in the liver, maintaining bone density and stimulation of the uterus during pregnancy. Oestrogen also acts on areas of the brain, helping to maintain body temperature and protect brain function and memory. There is however a negative aspect to oestrogen in that it some persons it promotes cancer, for example in the breast and uterus.. When oestrogen levels in the blood are reduced, as after the menopause, this can lead to osteoporosis, raised cholesterol levels with an increased risk of heart disease, poorer control of body temperature with resultant hot flushes and night sweats, and memory loss.

Oestrogen acts on cells in the body via nuclear proteins called oestrogen receptors (ER). The receptors bind to oestrogen, can change shape, form pairs and link to oestrogen response elements (ERE's) in certain genes. This attachment triggers formation of a transcription complex of co-activator proteins. This complex activates the gene(s) to induce a transcription enzyme, RNA polymerase, to transcribe messenger RNA, the

templates from which proteins are formed that induce the required change in the target cell.

Certain growth factors are also known to be important in the control and function of oestrogen receptors. The most frequently implicated growth regulators implicated in human cancers are members of the class 1 receptor tyrosine kinase family (erb B). Erb B tyrosine kinase receptors are over expressed in two-thirds of human breast cancers and are associated with malignant transformation. Included in this family of receptors are the HER-2 (erb B2) and HER-3 (erb B3) proteins which, together, constitute a high affinity functional receptor for heregulin (HRG), a ligand thought to be involved in the growth of breast epithelial cells. The HER-2 receptor promotes signal transduction along phosphorylation cascades. Phosphorylation of oestrogen receptors on tyrosine and/or serine residues has been associated with functional changes in hormone binding and nuclear localization. Tyrosine kinase inhibitors have been shown to block oestrogen-induced growth of breast cancer cells. It is also known that the oestrogen receptor can modulate HER-2 gene expression.

The expression of both the oestrogen receptor (ER positive) and HER-2 have important prognostic significance in hormone-dependent cancers, such as breast and prostate tumours. Over-expression of HER-2 is associated with the ER negative phenotype and a corresponding poor response to anti-oestrogen therapy. Similarly, a failure of anti-oestrogen treatment correlates with erb B receptor expression.

For many years it was believed that only one form of the oestrogen receptor existed, now called the alpha receptor (ERα). Using sucrose gradient centrifugation the ER obtained in the soluble fractions is recovered either as a large 8S complex or as a smaller 4S form. Furthermore, the 4S RT can be resolved into three components with isoelectric point (pI) values of 6.3, 6.6 and 6.8 while the 8S ER focuses as a single isoform at pI 6.1. Isoelectric focusing techniques, high performance liquid chromatography and DEAE-cellulose chromatography have also shown the ER to be present in various molecular forms.

In addition, the ER has an important relationship with the progesterone receptor (PgR) which has been identified in many tissues. There is considerable evidence that oestrogens induce the PgR gene through occupied ER binding to an ERE in the initiation region of the PgR gene. In breast cancers the response to endocrine therapy bears a strong relationship to the ER and PgR content of the cancer cells.

Breast cancers are highly heterogeneous, and show considerable variability in the profile of ER isoforms and their relationship to PgR levels. The ER isoform at pI 6.6 was present in 97% of ER positive breast cancers, the isoform at pI 6.1 in 83%, the pI 6.3 isoform in 39% of cancers and the pI 6.8 isoform in 33%, but there was evidence that for PgR expression all isoforms had to be present. Only 12% of cancers contained the full complement of ER isoforms. The ER isoforms at pI 6.1 and 6.8 were found only in PgR positive cancers. This heterogeneity in the ER isoform profile and the correlation with PgR levels might explain the variability in response to endocrine therapy.

Recently, Jan-Ake Gustafsson and co-workers (Proc. Natl. Acad. Sci.U.S.A. (1996); 93; 5925-5930) have described a second form of oestrogen receptor, and this has been termed the beta receptor (ERβ) to distinguish it from the original oestrogen receptor, now termed ER alpha (ERα). ERβ is highly homologous to ERα (J. Biol. Chem. (1997), 272; 25832-25838) and activates expression of reporter genes containing oestrogen response elements in an oestrogen-dependent manner. PCR analyses indicate that ERβ is highly expressed in prostate and ovary and with moderate expression in many other tissues including breast, uterus and testis. In addition, although ERβ and ERα share a high degree of homology, ERβ has certain important characteristics that are different from ERα. Tranxcription activation of AP1 enhancer elements by oestrogen receptors requires ligand and the AP1 transcription factors Fos and Jun. In studies (Paech K et al Science 1997; 277: 1508-1510) in which a luciferase reporter gene under control of an AP1 element was co-transfected with ERα in HeLa cells, all oestrogen agonists and antagonists examined were able to activate transcription. When ERβ was co-transfected instead of ERα, oestradiol (E_2) and diethylstilboestrol (DES) were unable to stimulate AP1-mediated transcription in the HeLa cells. Similar findings were reported in Ishikawa, MCF7 and MDA453 cells, although in these cell lines, E_2 and DES-liganded ERβ acted as antagonists at the AP1 site and inhibited raloxifene stimulation. Thus, when E_2 and DES bind to ERβ, they act as antagonists to oestrogen-responsive genes at the AP1 site. A splice variant of ERβ was described in 1998 (Peterson et al, Endocrinology 1998; 139: 1082-1092) and designated as ERβ2, with re-designation of the original as ERβ1. The affinity of ERβ2 for oestrogen is 35-fold lower than that of ERβ1 and the concentration of E_2 needed to produce half-maximal response with ERβ2 was approximately 1,000-fold greater than that for ERβ1.

Oestrogen receptor beta was first originated from a rat prostate cDNA library and its mRNA is prevalent in rat and human prostates. In the rat, ERβ levels are comparable to those in other highly expressing reproductive organs such as the ovary, endometrium and testis. By contrast, ERβ expression in the human prostate is low relative to testicular expression. ERβ is also regulated by androgens since in animals its mRNA is markedly decreased within 24 hr after castration, and the expression is restored rapidly with testosterone replacement. Findings from prostate cell lines indicate that ERβ is the key mediator of oestrogen-mediated events in the prostate. Of considerable interest is the finding that ERβ knockout mice show signs of prostatic hyperplasia with aging, which suggests that ERβ may protect against abnormal growth (Krege et al Proc. Natl. Acad. Sci. USA 1998; 95: 15677-15682).

A novel and unexpected aspect to the present invention is that trilostane and related compounds modulate principally the binding of ligand to the pI 6.3 isoform. Since ERβ corresponds, in molecular weight and positive immuno-reaction with a specific monoclonal antibody raised to ERβ receptor, to the pI 6.3 isoform, the modulation of ligand binding to the pI 6.3 isoform by trilostane and related compounds is therefore, by implication, modulation of the ERβ oestrogen receptor.

Another novel and unexpected aspect of the present invention is that binding of competitive ligands, such as the anti-oestrogens tamoxifen and raloxifene, to the oestrogen receptor is enhanced in the presence of trilostane and some of its related compounds, notably the major metabolite keto-trilostane.

Another novel and unexpected aspect of the present invention is that up-regulation of ERβ by trilostane and related compounds results in switching off the transcription of ERα in a breast cancer cell line (MCF7). Maruyama K et al (Biochem Biophys Res Commun 1998; 246: 142-147) reported that the presence of ERβ2 may suppress ERβ1 or ERα-mediated transcritional activity in response to oestrogen. More recently, Hall JM, & McDonnell DP *(Endocrinology* 1999 Dec;140(12):5566-78) determined that ERbeta functions as a transdominant inhibitor of ERalpha transcriptional activity at subsaturating hormone levels and that ERbeta decreases overall cellular sensitivity to estradiol. Additionally, they found that the partial agonist activity of tamoxifen manifest through ERalpha in some contexts was completely abolished upon coexpression of ERbeta. The anti-oestrogens, notably tamoxifen, have played an important role in the treatment of breast cancer. Tamoxifen has been in use for many years and has been

shown to have a significant response rate in ER positive breast cancer. The finding of a decrease in contralateral breast cancer incidence following tamoxifen administration for adjuvant therapy led to the concept that the drug might play a role in breast cancer prevention. A recent report of a study conducted by the National Surgical Adjuvant Breast and Bowel Project (*J Natl Cancer Inst* 1998 Sep 16;90(18):1371-88) showed tamoxifen reduced the risk of invasive breast cancer by 49% (two-sided P<.00001), with cumulative incidence through 69 months of follow-up of 43.4 versus 22.0 per 1000 women in the placebo and tamoxifen groups, respectively. The decreased risk occurred in women aged 49 years or younger (44%), 50-59 years (51%), and 60 years or older (55%); risk was also reduced in women with a history of lobular carcinoma in situ (56%) or atypical hyperplasia (86%) and in those with any category of predicted 5-year risk. Tamoxifen reduced the risk of noninvasive breast cancer by 50% (two-sided P<.002). Tamoxifen reduced the occurrence of estrogen receptor-positive tumors by 69%, but no difference in the occurrence of estrogen receptor-negative tumors was seen. However, an important side-effect was noted in that the rate of endometrial cancer was increased in the tamoxifen group (risk ratio = 2.53; 95% confidence interval = 1.35-4.97); this increased risk occurred predominantly in women aged 50 years or older. All endometrial cancers in the tamoxifen group were stage I (localized disease) and no endometrial cancer deaths occurred in this group. The ability of tamoxifen to stimulate the endometrium has been recognised for some time, and the formation of endometrial cancers is a worrying and unwanted complication. The endometrial stimulation is believed to be the result of an agonist effect on the ER alpha receptors in the uterus. However, it may also be due to blocking of both ER alpha and ER beta receptors in the uterus by tamoxifen and other This present invention suggests that trilostane and related compounds would block the endometrial stimulation caused by oestrogen antagonists such as tamoxifen and raloxifene and that the antagonists and trilostane could be used in combination to reduce the unwanted side-effects of the oestrogen antagonists.

Another widely recognised complication of anti-oestrogen therapy, and especially tamoxifen, for breast cancer is the development of drug resistance. The nature and cause of tamoxifen resistance is unknown but it may be due to the blockade of both ER alpha and ER beta. Oestradiol binding to ERβ at the AP1 site causes down-regulation of transcription, which is equivalent to "switching off" the cell's activity. Tamoxifen, on the other hand, activates transcription of ERβ at the AP1 site and thereby effectively "switches on" the cell's activity. This may be the basis for the well recognised partial

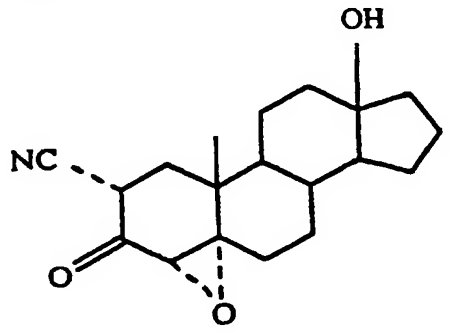
agonist action of tamoxifen and other similar compounds as well as their role as oestrogen antagonists.

Since trilostane increases oestradiol binding at the ERβ receptor this novel action of the drug and related compounds could have considerable significance for the treatment of certain hormone-dependent cancers including but not exclusively cancer of the breast, prostate and ovary.

The findings of the present invention also suggest that trilostane or its related compounds could be used in combination or sequentially with other hormonal agents or ligands, such as tamoxifen or raloxifene, in the treatment of certain cancers since trilostane appears to act on different oestrogen receptor isoforms to these agents.

Trilostane and Related Compounds

The present invention is concerned primarily with 2α-cyano-4α,5α-epoxyandrostan-17β-ol-3-one having the formula:



The compound of formula 1 has the common name "trilostane" and is described in British Patent Specification No. 1,123.770 and in the U.S. Patent Specification No. 3,296,295. These earlier Specifications describe the adrenocortical inhibiting properties of trilostane and related compounds, but this invention relates to a novel mode of action of trilostane and the related compounds and their use for the modulation of certain steroid receptor isoforms for the treatment of hormone-dependent cancers, such as cancers of the breast and prostate. In British Patent Specification No. 2,130,588 an improved method of manufacture for trilostane and related compounds was described.

This allowed the micronising of the compounds to particles having a mean equivalent sphere volume diameter of between 5-12μm, with at least 95% of the particles having a particle size of less than 50μm. The greater specificity of particle size improved the bio-availability of trilostane and controlled the amount of active metabolite formed thus improving the clinical response and decreasing variability of earlier preparations.

Trilostane has been extensively studied as a treatment of advanced breast cancer. Several published studies confirm the efficacy of trilostane with response rates of between 29% (Williams C.J.et al, Brit.J.Cancer (1993).68, 1210-1215) and 38% (Ingle J.N. et al, Am.J.Clin.Oncol., 1990, 13(2), 93-97). In all the previous work with trilostane it was believed that the mode of action was by competitive inhibition of the 3β-hydroxysteroid dehydrogenase enzyme system. The action was thought due to androgen depletion and hydrocortisone was given with trilostane to over-ride any feed-back mechanism. The compounds were shown to have no direct action on the then known oestrogen, androgen or progesterone receptors.

The basis of the present invention is that trilostane and related compounds have been shown to have a specific action on some oestrogen receptor isoforms and thereby modulate the receptor and the binding of oestrogen to the receptor(s). This invention has been made possible by the identification of receptor isoforms, whereas previous claims had been made on the belief, now known to be incorrect, that only one form of the oestrogen receptor existed. Thus, the present invention provides for a new role for trilostane and related compounds and allows for more specific and better-targeted therapy of hormone-dependant cancers and for the treatment of other diseases in which ERβ plays a role in the aetiology or progression of that disease.

METHODOLOGY

Using tissue, snap frozen at time of excision, cytosols were prepared by dismembration using a Braun II Mikrodismembrator. Frozen tissue was minced and pulverised. The resultant powder was resuspended in 1-mm phosphate buffer containing 10% (v/v) glycerol, 1.5mm EDTA, 10mM monothioglycerol, 20mM Sodium molybdate, aprotinin (1ug/ml) and STI (1ug/ml) and phenyl methyl sulfonyl fluoride (30ug/ml) at pH 7.4by stirring for 15 minutes at 4C. This suspension was centrifuged at 100,000g for 1 hour at 4C and the supernantant was used for competition analysis.

An aliquot of the supernatant was incubated with 3H oestradiol (5nM final concentration) in the presence or absence of either Trilostane at 10uM final

concentration or Tamoxifen at 10^{-7}M for 18 hours at 4C. In additional experiments an aliquot of the supernatant was incubated with tritiated tamoxifen (10^{-6}M) in the presence or absence of Trilostane (10^{-5}M) or keto-trilostane (Win 3280) (10^{-5}M) for 18 hours at 4C.

. After overnight incubation free steroid was separated from bound by incubating with dextran coated charcoal (0.25% w/v charcoal, 0.025% w/v Dextran T70 in 10mM Tris 1.5mM EDTA at pH 7.4) for 10 mins at 4C. The DCC was pelleted by centrifugation (10,000g for 10 mins) and aliquots of the supernatant were used for IEF separation.

For Scatchard analysis duplicate aliquots of the supernatants were incubated with increasing concentrations of 3H oestradiol in the presence and absence of a 100 fold excess of diethylstilboestrol. All tubes were then incubated in the presence or absence of trilostane (final concentration 10uM). After overnight incubation at 4C assay tubes were incubated with DCC as above to remove free steroid and aliquots of the resultant supernatant were incubated with toluene based scintillant and counted on a Scintillation Counter. Data obtained was analysed according to the method of Scatchard.

Isoelectric focusing (IEF) gels were cast in slabs of size 125 x 260 mm and separation was conducted along either the short side (short run) or the long side (long run) of the gel. Polyacrylamide gels (2 mm thick), containing 20% (v/v) glycerol and with high porosity (T=5%, C=3%) were used. A pH gradient of 5-10 was achieved using 1% (v/v) LKB ampholine 3.5-10 and 1.5% (w/v) LKB ampholine 5-8. Gels were photopolymerised at room temperature by means of a TR 26 polymerisation light, using riboflavin (0.004%) v/v) for at least 8 h. IEF was performed in a cold room and the temperature of the cooling water was kept constant at 4 oC using an LKB Multiphor II system with its chamber filled with 1M NaOH to minimise CO2 absorption from the air. Electrode solutions of 1M NaOH (cathode) and 1M H2SO4 (anode) were used. Gels were pre-focused for 40 min at 20 mA/20W/1200 V (short run).

After DCC extraction, aliquots (270ul) of the radioactive supernatant (3 mg protein/ml) derived from SSD assay were loaded near the cathode. The runs were carried out for 4 h using a 3000xi CC power supply at 2500 V/20 mA/20 W, constant power (long run) and at 1200 V/20mA/20 W, constant power, for 1.5 h (short run). A mixture of nine natural proteins (Biorad) was used for pH calibration. After the run the gels were cut into 2.5 mm slices and each slice was incubated with 5ml scintillation cocktail for 24h at room temperature and radioactivity assayed.

Electro-mobility shift assay (EMSA) was performed on MCF-7 cells cultured in serum-free medium for 5 days with two medium replacements to deplete the cells of endogenous estrogen. These serum-starved cells were then treated for 24 hours with either serum-free medium alone (control) or with serum-free medium containing □-estradiol (10^{-8}M), Trilostane (10^{-5}M), WIN 3280 (10^{-5}M), or Tamoxifen (10^{-6}M). 4 hours before harvesting this medium was replaced with fresh serum-free medium containing the corresponding addition, as above. Cells were then collected in phosphate-buffered saline (PBS) using a rubber policeman. Cell suspensions were centrifuged for 5 min at 4°C at 1000 X *g* then re-suspended in an equivalent volume of fresh ice-cold PBS and the centrifugation repeated. PBS was removed and cell pellets were re-suspended in extraction buffer (20 mM Hepes pH 7.8, 450 mM NaCl, 0.4 mM EDTA, 0.5 mM DTT, 25 % glycerol, 0.5 mM PMSF; Schreiber *et al.*, *Nucleic Acids Res.*, 1989, **17**: 6419). Samples were subjected to three freeze-thaw cycles using a dry ice-ethanol bath and 37°C water bath and then centrifuged for 10 min at 4°C at 10 000 X *g*. Cell extracts were transferred to fresh microtubes and stored at –80°C prior to use.

Oligonucleotides representing an estrogen response element sequence (ERE) were hybridised by heating to 80°C and allowing to cool to room temperature. The resultant double-stranded ERE was radiolabelled by end-labelling using Klenow Fragment and [□-^{32}P] dCTP (Amersham Pharmacia Biotech). Cell extracts were incubated for 30 min at room temperature in binding buffer (40 mM HEPES pH 7.4, 100 mM KCl, 2mM 2-mercaptoethanol, 20 % glycerol) containing 0.5 % bovine serum albumin and 50 □g poly[d(I-C)-d(I-C)]. After adding radiolabelled ERE (1 ng) extracts were incubated for a further 30 min at room temperature and then at 4°C for 30 min. 8 □l of cell extract was used per reaction. Samples were electrophoresed on a 6 % polyacrylamide native gel (pre-run for 30 min at 100 V) for 1 hour at 250 V at 4°C. After fixing the gel in 10 % acetic acid:30 % methanol for 15 min, gels were exposed to X-ray film overnight at –80°C with an intensifying screen.

The effect of trilostane on transcriptional activity in the MCF-7 breast cancer cell line was determined using the oestrogen reporterplasmid xTG.

The reporter plasmid xTG was designed so that oestradiol (E2) binding to native oestrogen receptor present within a cell would result in an accumulation of green fluorescent protein (GFP). XTG consists of a oestrogen response element. Upstream of a simple TATA promoter which together drive transcription of the GFP gene from the jellyfish *Aequorea victoria.*

The xERE sequence is made up of the following - GGTCA CAG TGACC TTGATTCAAAGTTAATGTAACCTC

The GFP gene - from GFPuv was supplied by Clontech (Clontech Laboratories, Inc., 1020 East Meadow Circle, Palo Alto, CA 94303-4230, USA)

MCF-7 cells were plated out in multiwell dishes and grown for 24 hours in serum free medium (MEM). Cells were then incubated in serum free medium or serum free medium containing oestradiol alone (10^{-8} M), trilostane alone (10^{-5}M) or oestradiol and trilostane together. Radiolabelled thymidine was added to each well (50μCi/ml) and cells were then cultured for a further 24 hours. At the end of this period the medium was aspirated and the cultured cells were rinsed 3-times with cold buffer solution(50mM Tris-HCl, pH 7.4). The cells were then dissolved in 1ml of 01.N NaOH and 0.5ml of this solution was mixed with 2.5ml of Scintillation cocktail and the intracellular radioactivity of ^{3}H was measured with a liquid scintillation counter.

RESULTS

In the IEF experiments described above Trilostane (10^{-5}M) modulated the binding of oestrogen onto all the oestrogen receptor isoforms but principally onto the pI 6.3 isoform. By contrast tamoxifen (10^{-6} M) displaced oestrogen from all the oestrogen receptor isofroms but principally the pI 6.1 isoform.

In experiments studying the effect of trilostane on oestradiol binding kinetics *in rat uterus* we observed an altered affinity of receptors to steroid in that the kD changed from a lower affinity to a higher affinity while in rat prostate we observed an increase in receptor concentration.

Scatchard analysis performed in the presence of the major metabolite, keto-trilostane, also altered the kD to a higher affinity. However the presence of the stage III compound had no effect.

These data reflect the ability of trilostane and related compounds to modulate certain functions and characteristics of the oestrogen receptor, such as the ability to modulate ligand binding to individual isoforms, receptor protein conformation, receptor dimerisation and binding co-operativity. These results are a novel and surprising finding for trilostane and related compounds.

The pI 6.3 isoform of the oestrogen receptor was identified as the product of the ERβ gene on the basis of its molecular weight and positive immune reaction with a specific

monoclonal antibody raised to the ERβ receptor. The pI 6.6 isoform corresponds with the ERα gene product.

Figure 6 shows the detection of bands which are characteristic of the formation of complexes between estrogen receptor (ER) present in the MCF-7 cell extracts and the radiolabelled ERE. These complexes could be competed out by co-incubation with a 50-fold molar excess of unlabelled ERE but not by 50-fold excess of a probe representing the AP1 gene sequence, thus demonstrating the specificity of the ER-ERE interaction in this system.

In the absence of ligand (lane 1) ER-ERE complexes are apparent. However, extracts from cells treated with radio-labelled estradiol (lane 2) showed an increased level of ER-ERE complex formation (110 % of control density corrected for protein concentration in the extract. Treatment with Trilostane (lane 3) reduced complex formation to 96 % of control levels (lane 1), while WIN 3280 (lane 4) dramatically reduced ER-ERE complex formation to 68 % of control. Tamoxifen also reduced complex formation (lane 5) (93 % of control).

These data suggest that both Trilostane and keto-trilostane (WIN 3280) are able to modulate the conformation of the estrogen receptor in such a way as to reduce the strength of interaction between this receptor and its response element. This clearly has profound implications with respect to the ability of estrogen to activate specific genes in breast cancer cells. These compounds would be expected, therefore, to reduce the level of transcription of key estrogen-responsive genes in these cells.

Results using the cell line MCF-7, transiently transfected with xTG, suggest that while E2 enhances production of GFP the antiestrogens tamoxifen and raloxifene reduced E2-driven transactivation. E2 was used at a concentration of 10-8M while all other compounds were used at 10-6M. All ligands were added to cell media during transfection. Under the same conditions trilostane also reduced transcription of GFP.

Cells grown in the presence of oestradiol (E2) show a 15% increase in thymidine labeling ($p<0.01$) when compared to control, which is completely blocked by the presence of trilostane (E2+T). In addition, trilostane alone inhibits thymidine uptake of MCF-7 cells by 31% compared with control ($p<0.01$).

LEGENDS

Figure 1. shows the action of trilostane at different concentrations on tritiated oestradiol (5nM) binding to the pI 6.3 isoform (ERβ) of the oestrogen receptor in rat uterus. The graph shows tritiated oestradiol (in DPM) recovered in sequestered slices from IEF gel (see text).

Figure 2. Scatchard plots for the tritiated oestradiol binding to rat uterine oestrogen receptors in the absence (open symbols) and presence (closed symbols) of trilostane at 10μm. Presence of trilostane increases the apparent affinity of oestrogen binding (given by the slope of the regression lines) and the concentration of total receptor (given by the X axis intercept).

Figure 3. Scatchard plots for the tritiated oestradiol binding to rat prostate oestrogen receptors in the absence (open symbols) and presence (closed symbols) of trilostane at 10μM. Presence of trilostane appears to increase the concentration of total receptor (given by the x axis intercept).

Figures 4. Scatchard plots for the tritiated oestradiol binding to rat uterus oestrogen receptors a) for control i.e. no additional compounds, and in the presence of (b) trilostane, (c) stage III compound and (d) keto-trilostane. Presence of either trilostane or keto-trilostane increased the apparent affinity of oestrogen binding (given by the slope of the regression lines), but no change in binding affinity was found with the stage III compound.

Figure 5. Effect of a) trilostane and b) keto-trilostane (Win 3280) on tritiated tamoxifen binding to oestrogen receptor isoforms in rat uterus. The graphs show increased tritiated tamoxifen binding to oestrogen receptor isoforms in the presence of either Trilostane (10^{-6}M) or keto-trilostane (10μM).

Figure 6 Electrophoretic mobility shift assay showing characteristic double band shift resulting from binding of estrogen receptor present in MCF-7 breast cancer cell extracts to radiolabelled estrogen response element run on 6 % native polyacrylamide gel. Serum-starved MCF-7 cells were treated for 24 hours with the following treatments which were replenished 4 hours prior to harvesting of cells for extract preparation.

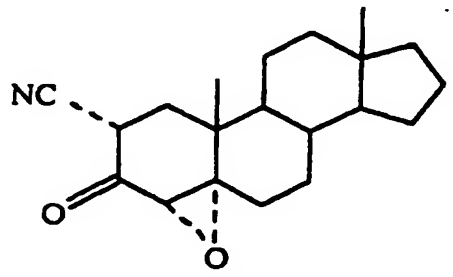
Lane 1 = serum-free medium alone
2 = serum-free medium + estradiol (10^{-8}M)
3 = serum-free medium + Trilostane (10^{-5}M)
4 = serum-free medium + WIN 3280 (10^{-5}M)
5 = serum-free medium + Tamoxifen (10^{-6}M)

Figure 7. Thymidine labeling of MCF-7 cells incubated for 24 hours in the presence and absence of Oestradiol (10^{-8} M) and trilostane (10□M). Cells grown in the presence of oestradiol (E2) show a 15% increase in thymidine labeling ($p<0.01$) when compared to control, which is completely blocked by the presence of trilostane (E2+T). In addition, trilostane alone (T) inhibits thymidine uptake of MCF-7 cells by 31% compared with control ($p<0.01$).

Figure 8. MCF-7 cells transiently transfected with xTG for 3 days: (a) E2 only showed GFP accumulation, (b) E2 plus trilostane showed blue-green auto fluorescence from the transfection reagent only.

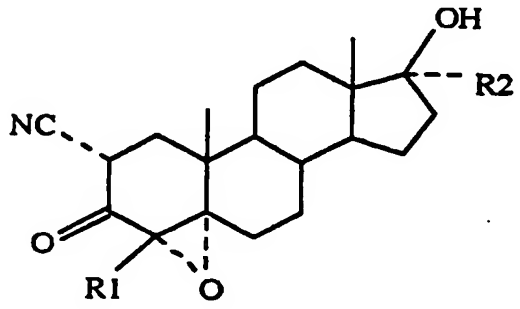
CLAIMS

1. A method whereby a 2 α-cyano-4 α, 5 α–epoxy-3-oxo-steroid compound having a basic ring structure of the general formula given below is used to modulate the binding of oestrogen to certain isoforms of the oestrogen receptor in human and animal cells, notably the oestrogen receptor beta.



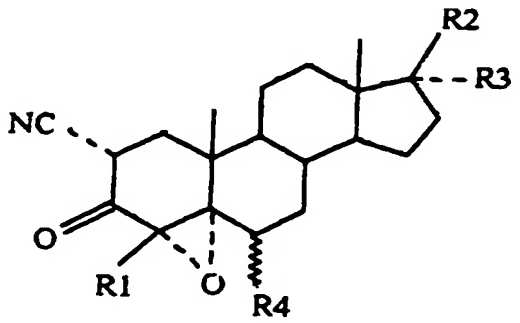
2. A particulate compound according to claim 1 in which the steroid moiety has from 19-23 carbon atoms.

3. A particulate compound according to claims 1 having the general formula:



Where R1 is hydrogen or methyl and R2 is hydrogen or lower alkyl.

4. A particulate compound according to claim 1 having the general formula:



Wherein R^1 is hydrogen or methyl;
R^2 is hydroxy or lower-alkanoyloxy;
R^3 is hydrogen, lower-alkyl, lower-alkenyl or lower-alkynyl;
R^2 and R^3 together represent oxo or ethylenedioxy;
R^4 is hydrogen or methyl;
or 3-enol lower-alkanoate esters thereof;
with the proviso that when R^1 is hydrogen, R^4 is α-methyl, and when R^1 is methyl, R^4 is hydrogen or β-methyl.

5. A particulate compound according to claims 1 - 4 wherein R^1 and R^2 are hydrogen.

6. A particulate compound according to claims 1 - 4 wherein R^1 and R^2 are methyl.

7. The compound of the structure according to any of the preceding claims being in particulate form and consisting of particles having a mean equivalent sphere volume diameter of about 12 μm or less, at least 95% of the particles having a particle size of less than about 50 μm.

8. A particulate compound according to any one of the preceding claims, wherein the particles have a mean equivalent sphere volume diameter of about 5 to about 12 μm.

9. A particulate compound according to any one of the preceding claims, wherein the particles have a mean equivalent sphere volume diameter of less than 5μm.

10. A particulate compound according to any one of the preceding claims, wherein its cumulative percentage oversize versus size characteristic curve exhibits a standard deviation of about 2 μm.

11. A particulate compound according to any one of the preceding claims, wherein its

specific surface area is about 2 - 5 m^2g^{-1} or higher.

12. A method using particulate compounds with a structure according to claims 1 to 6 and/or the particle size is as defined in any one of claims 6 to 11 that modulate ligand binding to specific oestrogen receptor sub-types in human and animal cells.

13. A method using particulate compounds with a structure according to claims 1 to 6 and/or the particle size is as defined in any one of claims 6 to 11 that modulate ligand binding to specifically to the oestrogen receptor beta (ERβ) sub-type in human and animal cells.

14. A method using particulate compounds with a structure according to claims 1 to 6 and/or the particle size is as defined in any one of claims 7 to 8 that modulate specific oestrogen receptor sub-types in human and animal cells and human and animal cancer cells including but not exclusively, cancer of the breast, prostate and uterus.

15. A method using particulate compounds according to any of the preceding claims for the treatment of human and animal diseases by modulation of specific oestrogen receptor sites notably ERβ to affect binding of hormones or other ligands.

16. A method using particulate compounds according to any of the preceding claims for the treatment of human and animal diseases in which oestrogen receptor beta plays a role in the aetiology or progression of the disease.

17. A method using particulate compounds according to any of the preceding claims to modulate ligand binding to oestrogen receptor beta for the enhancement of any or all functions of cells or organs in which oestrogen receptor beta plays a role in those functions.

18. A pharmaceutical composition, which composition comprises a particulate compound according to any of the preceding claims and one or more pharmaceutically-acceptable excipients or carriers in proportions necessary to achieve acceptable formulation.

19. A composition according to preceding claims in the form of a tablet, a capsule, a granulate for suspension, a cream, an ointment, a suppository or a suspension.

20. A composition according to any one of the preceding claims in unit dosage form and comprising a unit dosage of from about 50 to 250 mg of the compound.

21. A composition according to preceding claims and substantially as hereinbefore described with reference to any one of the specific examples.

22. A particulate compound according to claims 1 to 6 and of particulate size and/or surface area according to claims 7 to 11 and having the actions according to claims 12 to 17 and having the composition according to claims 18 to 21 and also in combination with agents and compounds, such as but not exclusively tamoxifen or raloxifene, that competitively

inhibit oestrogen binding to receptors and also in combination with other agents and compounds that bind to or specifically modulate the oestrogen receptor in human and animal cells.

23. A particulate compound according to claims 1 to 6 and of particulate size and/or surface area according to claims 7 to 11 and having the actions according to claims 12 to 17 and having the composition according to claims 18 to 22 used in combination or sequentially with agents and compounds, such as but not exclusively tamoxifen or raloxifene, in order to enhance the activity of such compounds or agents by increasing binding of such compounds or agents to receptor or inhibiting transcriptional activity.

24. A particulate compound according to claims 1 to 6 and of particulate size and/or surface area according to claims 7 to 11 and having the actions according to claims 12 to 17 and having the composition according to claims 18 to 22 used in combination or sequentially with agents and compounds, such as but not exclusively tamoxifen or raloxifene, in order to block or suppress or diminish unwanted side-effects of such agents or compounds.

25. A particulate compound according to claims 1 to 6 and of particulate size and/or surface area according to claims 7 to 11 and having the actions according to claims 12 to 17 and having the composition according to claims 18 to 22 used in combination or sequentially with agents and compounds, such as but not exclusively tamoxifen or raloxifene, in order to reverse the development of resistance to such agents and compounds in normal or diseased tissues or organs.

Application No: GB 0000984.5 **Examiner:** Diane Davies
Claims searched: 1, 11-20 and 21-25 (in part) **Date of search:** 22 May 2000

Patents Act 1977
Search Report under Section 17

Databases searched:

UK Patent Office collections, including GB, EP, WO & US patent specifications, in:

UK Cl (Ed.R): A5B

Int Cl (Ed.7): A61K 31/58

Other: Online databases: CAS-ONLINE, EPODOC, JAPIO, TXTE, WPI

Documents considered to be relevant:

Category	Identity of document and relevant passage	Relevant to claims
X	EP 0296097 A (Schering AG) Whole document: use of a progesterone synthesis inhibitor of the trilostane or epostane type for treating *inter alia*, hormone dependant tumours.	At least claim 1
A	GB 2130588 A (Sterwin AG) Whole document: preparation of steroid compounds such as trilostane in particulate form.	
X	US 5372996 A (Endorecherche Inc.) See in particular Example 1 disclosing trilostane and epostane in the treatment of prostrate cancer.	At least claim 1
X	Anticancer Res., 15(4), 1349-54, (1995) A.A.Geldof et al, "Inhibition of 3.beta.-hydroxysteroid dehydrogenase: An approach for prostate cancer treatment?".	At least claim 1
X	J. Steroid Biochem. Mol. Biol., 37(2), 231-6, (1990) M. Takahashi *et al*, "Inhibitory effect of synthetic progestins, 4-MA and cyanoketone on human placental 3.beta.-hydroxysteroid dehydrogenase/5→4-ene-isomerase activity".	At least claim 1

X	Document indicating lack of novelty or inventive step	A	Document indicating technological background and/or state of the art.
Y	Document indicating lack of inventive step if combined with one or more other documents of same category.	P	Document published on or after the declared priority date but before the filing date of this invention.
&	Member of the same patent family	E	Patent document published on or after, but with priority date earlier than, the filing date of this application.

Application No: GB 0000984.5 **Examiner:** Diane Davies
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Category	Identity of document and relevant passage	Relevant to claims
X	Cancer Treatment Reports, 71(12), 1197-1201, (1987) C.J. Williams *et al*, "Multicenter study of Trilostane: A new hormonal agent in advanced postmenopausal breast cancer".	At least claim 1
X	Cancer Chemo. Pharmacol., 10(3), 158-60, (1983) C.G. Beardwell *et al*, "Trilostane in the treatment of advanced breast cancer".	At least claim 1

X	Document indicating lack of novelty or inventive step	A	Document indicating technological background and/or state of the art.
Y	Document indicating lack of inventive step if combined with one or more other documents of same category.	P	Document published on or after the declared priority date but before the filing date of this invention.
&	Member of the same patent family	E	Patent document published on or after, but with priority date earlier than, the filing date of this application.